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Core 2 GlcNAc-T Inhibitors

The present invention relates to the use of known and novel compounds as inhibitors of UDP-GlcNAc:Galβ1,3GalNAc-R (GlcNAc to GalNAc) β-1,6-N-acetylglucosaminyl transferase (core 2 β-1,6 N-acetylaminotransferase, core 2 GlcNAc-T -EC 2.4.1.102).

Such inhibitors have applications in therapy for diseases associated with raised activity of core 2 GlcNAc-T, in particular inflammatory diseases, athero-sclerosis, diabetic cardiomyopathy, cancers – including treatment or prevention of metastasis – or diabetic retinopathy.

The present inventors have determined that the compounds herein described can inhibit glucose-induced activity of core 2 GlcNAc-T and glucose induced binding of human leukocytes to cultured bovine retinal capillary endothelial cells as measured in assays described herein. The administration of these compounds, hereinafter referred to as Core 2 GlcNAc-T inhibitors to patients can prevent or treat the abnormal formation of core 2 O-glycans and sialyl Lewis^x by inhibiting raised activity of core 2 GlcNAc-T in the aforementioned disease states.

Following initiation of glycosylation by the attachment of an N-acetyl-glucosamine (GalNAc) to either a serine or threonine residue in a protein to be glycosylated, processing proceeds by elongation, branching and then terminal modification of the O-glycans.

Essential steps in O-glycan elongation and branching are catalysed by multiple glycosyl transferase isoforms from families of homologous glycosyl-transferases. Depending on which saccharide groups are subsequently attached to this first GalNAc residue, O-glycans are divided into four major subtypes (Figure 1). The core 1 structure is formed by addition of galactose to form Galβ1-3GalNAc-αSer/Thr. The core 2 structure requires the core 1 structure as substrate and is formed by addition of GlcNAc to form Galβ1-3(GlcNAcβ1-6)GalNAc-αSer/Thr. The core 3 structure is formed by the addition of GlcNAc to form GlcNAcβ1-3GalNAc-αSer/Thr. The core 4 structure requires the core 3 structure as substrate and is formed by addition of GlcNAc to form GlcNAcβ1-3(GlcNAcβ1-6)GalNAc-αSer/Thr. Other modifications to the core GalNAc structure have also been found, but appear to be uncommon. All these core structures are further modified by galactosylation, sialylation, fucosylation, sulfation or elongation to eventually form the O-glycan.

Three forms of Core 2 GlcNAc-T are known. Core 2 GlcNAc-T I identified in from leukemic cells, core 2 GlcNAc-T II identified in mucin secreting tissue, and a third thymus associated type designated core 2 GlcNAc-T III.

Cell surface O-glycans are known to play a crucial role in mediating cell–cell interactions in development and certain disease states. The patterns of protein glycosylation are determined largely by the activity and specificity of the glycotransferase enzymes, such as core 2 GlcNAc-T which is expressed in the Golgi apparatus (1-2). Core 2 GlcNAc-T plays a crucial role in the biosynthesis of O-linked glycans (3-4) and represents an important regulatory step for the extension of O-linked sugars with polylactosamine (i.e. repeating Galβ1-4GlcNAcβ1-3), a structure associated with malignant transformation (5-6).

Changes in the activity of core 2 GlcNAc-T have been associated with various disease states, such as T-cell activation, cancers, metastasis, myeloblastic leukaemia, myocardial dysfunction and inflammation (7-18). Regulation of core 2 GlcNAc-T is thought to be particularly important, because addition of lactosamine structures to the basic core oligosaccharides formed by this enzyme and subsequent modification with fucose and sialic acid, results in the formation of the Lewis^x, sialyl-sialyl Lewis^a, and Lewis^x sugar groups that constitute the ligands of selectins which are cell adhesion proteins. This selectin-ligand interaction plays an important role in many processes.

Inflammation is how the body generally responds to infection or to some other form of trauma. One of the major events during inflammation is the movement of cells of the immune system from the blood stream to the infected or injured area. Once at the site of injury, these cells are responsible for the isolation, destruction and removal of the offending agent .

Acute inflammation, characterised by short duration (minutes to days), is essential for health, but sometimes the inflammatory process does not end when appropriate, and it is this that causes problems. Chronic inflammation is characterised by long duration (days, weeks, months and even years), lymphocytes and macrophages, tissue destruction and repair, and vascular proliferation and fibrosis. Inflammation can also be triggered inappropriately by the body's normal constituents and plays a role in common diseases, such as asthma, rheumatoid arthritis and inflammatory bowel disease.

Many cell adhesion molecules are known to be involved in the process of

inflammation. At the site of inflammation, leukocytes first adhere to the vascular endothelial cells prior to the extravasation process. It is postulated that selectins play a crucial role in the initial adhesion of leukocytes to endothelial cells. Cell adhesion mediated by selectins and their carbohydrate ligands leads to the tethering and rolling of leukocytes on endothelial linings. This then leads to the secondary firm adhesion. Within hours of the initial stimulus, neutrophils begin to enter the tissue and may continue transmigration for many days. In some inflammatory conditions, tissue damage is caused by direct injury of the vessels and amplified by the subsequent recruitment of neutrophils into the tissue.

The expression of O-glycans reduces cell-cell interactions because of the bulkiness of these adducts. The expression of core 2 O-glycans is regulated by the transcriptional levels of core 2 GlcNAc-T in all of these cases. Antigen-mediated activation of peripheral T and B-cells is characterised by increased activity of core 2 GlcNAc-T and branched O-glycans on CD43 (leukosialin) (19-20).

Leukocyte extravasation, lymphocyte trafficking and other processes involve O-glycan synthesised by core 2 GlcNAc-T. Specifically, cell-surface O-glycan structures terminating in sialyl Lewis^x are involved in the recruitment of leukocytes to the site of inflammation. Core 2 GlcNAc-T is not important for T-cell development, but over expression of this enzyme has been shown to completely block the development of myeloid lineages. Over expression of core 2 O-glycans has also been reported to affect the interaction between T-cells and B-cells (TB interaction). This T-B interaction is crucial for humoral immune response and is mediated through binding of the CD40 ligand (CD40L) on T-cells with CD40 on B-cells (CD40L-CD40 interaction). This interaction induces the proliferation of B-cells. Over expression of core 2 O-glycans has been shown to cause significant reduction in CD40L-CD40 interaction (21).

It is possible to effectively block the initial step of leukocyte invasion from taking place, by blocking the synthesis of sialyl Lewis^x on the cell surface of activated leukocytes and thereby halting their interactions with selectins. Therefore, inhibitors of core 2 GlcNAc-T that can reduce the activity of core 2 GlcNAc-T have utility in modulating inflammation.

Atherosclerosis is a progressive inflammatory disease of unknown mechanism. Recruitment and adhesion of circulating leukocytes to the endothelium particularly at arterial branches and bifurcations is one of the earliest events known to occur in atherogenesis. Integrins on the leukocytes then cause a stronger attachment

between the cells. Leukocytes transmigrate through into the sub-endothelial space where they begin to accumulate in the intima. Monocytes become converted to activated macrophages with the presence of oxidised low density lipoprotein (LDL - oxLDL), these activated macrophages take up the modified types of lipoprotein via their scavenger receptors and differentiate to become foam cells. Histological analysis of atherosclerotic coronary arteries from patients who died of acute coronary syndromes demonstrate foam cells, macrophages, lymphocytes and mast cells were present in unstable or ruptured plaques (49).

At least three leukocyte adhesion molecules, E-selectin, ICAM-l, and VCAM-l, have been identified in human atherosclerosis (50,51). Further, in contrast to normal vessels, P selectin is overly expressed by epithelial cells in atherosclerotic lesions and expression of E-selectin (52) and ICAM-l (53) at the arterial lumen, has been found to be increased in arterial segments with mononuclear leukocyte accumulation. A third adhesion molecule, VCAM-l, has been detected in animal models of atherosclerosis, and also has been shown to be more prevalent in the intima of atherosclerotic plaques than in non atherosclerotic segments of human coronary arteries.

Chibber *et al* (54) evaluated the importance of core 2 GlcNAc-T in increased leukocyte-endothelial cell adhesion and found significant increases in the activity of this enzyme in leukocytes of diabetic patients. However, until now there has been no evidence that core 2 GlcNAc-T activity is raised in circulating leukocytes of patients suffering from atherosclerosis. The applicants have now demonstrated that activity of the enzyme Core 2 GlcNAc-T is indeed raised in circulating leukocytes from patients with atherosclerosis, suggesting that compounds capable of lowering the activity of core 2 GlcNAc-T would be useful in the treatment or prevention of atherosclerosis or in preventing reoccurrence of atherosclerotic plaques in patients following interventions.

Although the clinical symptoms of diabetic cardiomyopathy have been identified, its pathogenesis is uncertain. The definition of diabetic cardiomyopathy describes both specific defects in the diabetic's myocytes, such as fibrosis leading to myocardial hypertrophy and diastolic dysfunction, and associated changes in the heart which have developed during the course of diabetes.

There is now strong evidence suggesting that raised activity of core 2 GlcNAc-T is directly responsible for elevated glycoconjugates, commonly observed in the heart tissue of diabetic animals and patients. In support of this, it has recently

been shown that increased core 2 GlcNAc-T activity causes pathology similar to that observed in the heart of diabetic patients after years with the condition, in the heart of diabetic experimental animal models. Studies were carried out using a transgenic mouse with core 2 GlcNAc-T expression driven by a cardiac myosin promoter. At 4 months, a marked hypertrophy of the left ventricle and general hypertrophy of the heart was observed (16-17).

Marked changes in core 2 branching and core 2 GlcNAc-T activities are associated with malignant transformation, leukaemia and carcinomas (21, 33-36). Rat fibroblasts and mammary carcinoma cells transfected with T24H-ras express core 2 O-glycans as they become metastatic tumours (33).

There is a great deal of evidence pointing to the involvement of core 2 GlcNAc-T in cancer and cancer metastasis. For example, highly metastatic colonic carcinoma cells both express more sialyl Lewis^x than their low metastatic counterparts and adhere more strongly to E-selectin than poorly metastatic cells. There is a strong correlation between the expression of sialyl Lewis^x in tumour cells and tumour progression (34). Moreover, a good correlation exists between the expression of sialyl Lewis^x in core 2 O-glycans and lymphatic and venous invasion.

Recent findings suggest that core 2 GlcNAc-T in combination with αl,3-Fuc-T contributes to the selectin-mediated metastasis in oral cavity carcinomas (35). Moreover, Western blot analysis revealed the presence of a major approximately 150 kDa glycoprotein that carries a-linked oligosaccharides recognised by anti-sLe^x monoclonal antibody in sLe^x -positive pre-B leukaemia cell lines. This correlation of core 2 GlcNAc-T with CD15 expression suggests that core 2 GlcNAc-T is a regulator of the cell surface expression of sialyl Lewis^x in human pre-B lymphoid cells. These results indicate that core 2 GlcNAc-T mRNA detected by *in situ* hybridisation reflects the malignant potentials of pulmonary adenocarcinoma, because lymph node metastasis is the most affecting factor to the patient's prognosis.

Expression of sialyl Lewis^x in mouse melanoma Bl6-FI by transfection with the enzyme 1,3-fucosyltransferase have also confirmed the importance of sialyl Lewis^x in tumour metastasis. Intravenous injection of the transfected cells into mice formed a large number of lung tumour nodules, while the parent B16-FI cells scarcely formed tumours.

The expression of sialyl Lewis^a, sialyl Lewis^x (both selectin ligand

carbohydrate structures) and raised activity of core 2 GlcNAc-T are all closely associated with malignancy of colorectal cancer (36). Recently, Numahata (37) demonstrated that sialyl Lewis^x expression in primary bladder carcinoma is a predictor of invasive and metastatic outcome. No other carbohydrate epitope examined to date has equal prognostic value. Recently US 2004/0033521 disclosed that core 2b GlcNAc-T is over expressed in both liver and stomach tumours and in colon cancer and liver metastasis samples. Furthermore, WO 04/093662 demonstrates that core 2 GlcNAc-T is raised in prostate cancer testicular and bladder cancer. Levels of core 2 GlcNAc-T increase with increasing chance of metastasis or recurrence of disease.

Accordingly inhibitors of core 2 GlcNAc-T would be expected to reduce the production of the O-glycans, for example those bearing sialyl Lewis^x , and would reduce cancer invasiveness and metastasis and be useful in treatment of cancers where core 2 GlcNAc-T expression is raised above normal levels for that tissue type.

Diabetic retinopathy is a progressive vision threatening complication of diabetes (38) characterised by capillary occlusion, formation of microvascular lesions and retinal neovascularisation adjacent to ischaemic areas of the retina (39-40).

It has recently been found that raised activity of core 2 GlcNAc-T is directly responsible for increased leukocyte-endothelial cell adhesion and capillary occlusion in diabetic retinopathy (41). It has now also been demonstrated that elevated glucose and diabetic serum increase the activity of core 2 GlcNAc-T and the adhesion of human leukocytes to endothelial cells. This occurs through PKCβ2-dependent phosphorylation of core 2 GlcNAc-T (42-43). This regulatory mechanism involving phosphorylation of core 2 GlcNAc-T is also present in polymorphonuclear leukocytes (PMNs) isolated from Type 1 and Type 2 diabetic patients.

Inhibition of PKCβ2 activation by the specific inhibitor, LY379196, attenuates serine phosphorylation of core 2 GlcNAc-T, prevents the increase in activity and thus prevents increased leukocyte-endothelial cell adhesion. Such an inhibitor provides validation that reduction of core 2 GlcNAc-T activity provides a method of preventing increased leukocyte-endothelial cell adhesion and preventing capillary occlusion in retinopathy associated with diabetes or hyperglycaemia.

Fenugreek has been used for thousands of years for the treatment of diabetes. The plant contains many active ingredients, such as coumarins, saponins and

glycosides, Many studies (44) have demonstrated the hypoglycaemic properties of fenugreek in both animals and humans. The hypoglycaemic properties have been attributed to the amino acid 4-hydroxyisoleucine which has potent insulinotropic activity (45-46).

The present inventors have now determined that certain compounds are inhibitors of Core 2 GlcNAc-T. Certain of these compounds are obtainable from fenugreek seeds and from other plant sources.

In a first aspect of the invention is provided a method of treatment of conditions associated with raised activity of the enzyme core 2 GlcNAc-T comprising administration of an effective amount of a compound of the formula I to a patient in need thereof. Preferably, the disease is an inflammatory disease, asthma, rheumatoid arthritis, inflammatory bowel disease, diabetic cardiomyopathy, myocardial dysfunction, cancer, cancer metastasis or diabetic retinopathy.

Cancers include leukaemia, oral cavity carcinomas, pulmonary cancers such as pulmonary adenocarcinoma, colorectal cancer, bladder carcinoma, liver tumours, stomach tumours colon tumours, prostate cancer, testicular tumour, mammary cancer, lung tumours oral cavity carcinomas and any cancers where core 2 GlcNAc-T expression is raised above normal levels for that tissue type.

Preferably the core 2 GlcNAc-T inhibitor comprises a sugar-derived substituent. The term sugar-derived substituent means a saccharide, in which optionally one or more hydrogens and/or one or more hydroxyl groups have been replaced by -R, -OR, -SR, -NR wherein R is methyl, ethyl or propyl to form a derivative.

Saccharides include, but are not limited to, monosaccharides, disaccharides, trisaccharides, tetrasaccharides and polysaccharides.

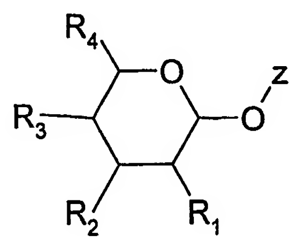
Monosaccharides include, but are not limited to, arabinose, xylose, lyxose, ribose, glucose, mannose, galactose, allose, altrose, gulose, idose, talose, ribulose, xylulose, fructose, sorbose, tagatose, psicose, sedoheptulose, deoxyribose, fucose, rhamnose, 2-deoxy-glucose, quinovose, abequose, glucosamine, mannosamine, galactosamine, neuraminic acid, muramic acid, N-acetyl-glucosamine, N-acetyl-mannosamine, N-acetyl-galactosamine, N-acetylneuraminic acid, N-acetylmuramic acid, O-acetylneuraminic acid, N-glycolylneuraminic acid, fructuronic acid, tagaturonic acid, glucuronic acid, mannuronic acid, galacturonic acid, iduronic acid, sialic acid and guluronic acid.

Preferably, the core 2 GlcNAc-T inhibitor comprises at least one sugar-

derived substituent; more preferably, the core 2 GlcNAc-T inhibitor comprises at least two sugar-derived substituents.

Preferably, each sugar-derived substituent is independently a mono-, di-, tri- or tetrasaccharide; more preferably, each sugar-derived substituent is independently a mono- or trisaccharide.

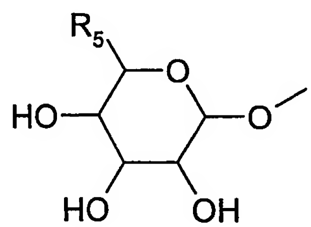
Preferably, the core 2 GlcNAc-T inhibitor is a compound of the formula I



I

wherein R_1 is -OH, C_{1-6} alkoxy, $-NR_8R_9$, or a monosaccharide of the formula:

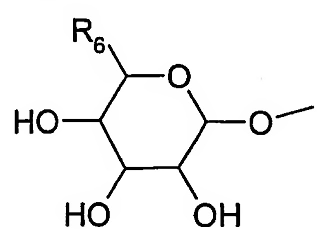
IIa:



IIa

Preferably R_1 is -OH, $-NR_8R_9$, or a monosaccharide of the formula IIa; more preferably R_1 is $-NR_8R_9$, or a monosaccharide of the formula IIa; most preferably R_1 is a monosaccharide of the formula IIa;

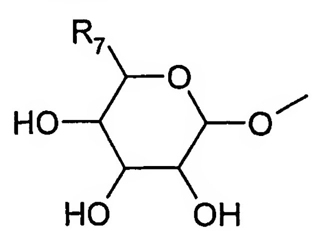
R_2 is –OH, C_{1-6} alkoxy or a monosaccharide of the formula IIb:



IIb

Preferably R_2 is –OH or a monosaccharide of the formula III; more preferably R_2 is –OH or a monosaccharide of the formula III; most preferably R_2 is –OH;

R_3 is -OH, C_{1-6} alkoxy or a monosaccharide of the formula IIc:



IIc

Preferably R_3 is -OH or a monosaccharide of the formula IIc; more preferably

R_3 is a monosaccharide of the formula IIc; most preferably R_3 is glucose;

R_4 is C_{1-6} alkyl, C_{1-6} hydroxyalkyl or C_{1-6}-alkoxy-C_{1-6}-alkyl; preferably R_4 is C_{1-6} alkyl or C_{1-6} hydroxyalkyl; more preferably R_4 is $-CH_2OH$ or $-CH_3$; most preferably R_4 is $-CH_2OH$;

R_5 is C_{1-6} alkyl, C_{1-6} hydroxyalkyl or C_{1-6}-alkoxy-C_{1-6}-alkyl; preferably R_5 is C_{1-6} alkyl or C_{1-6} hydroxyalkyl; more preferably R_5 is $-CH_3$, $-C_2H_5$, $-CH_2OH$ or $-C_2H_4OH$; most preferably R_5 is $-CH_3$;

R_6 is C_{1-6} alkyl, C_{1-6} hydroxyalkyl or C_{1-6}-alkoxy-C_{1-6}-alkyl; preferably R_6 is C_{1-6} alkyl or C_{1-6} hydroxyalkyl more preferably R_6 is $-CH_2OH$ or $-CH_3$; most preferably R_6 is $-CH_2OH$;

R_7 is C_{2-6} alkyl, C_{1-6} hydroxyalkyl or C_{1-6}-alkoxy-C_{1-6}-alkyl; preferably R_7 is C_{1-6} hydroxyalkyl or C_{1-6}-alkoxy-C_{1-6}-alkyl; more preferably R_7 is $-CH_2OH$ or C_{1-6} alkoxymethyl; most preferably R_7 is $-CH_2OH$;

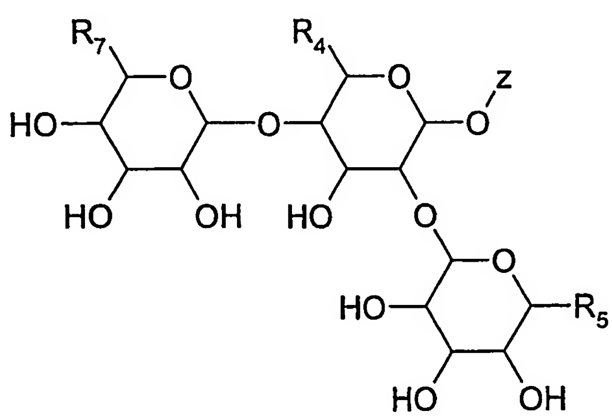
R_8 is H, C_{1-6} alkyl or C_{1-6} acyl; preferably R_8 is H or C_{1-6} alkyl; more preferably R_8 is H or CH_3; most preferably R_8 is H;

R_9 is H, C_{1-6} alkyl or C_{1-6} acyl; preferably R_9 is H or C_{1-6} acyl more preferably R_9 is H or $-COCH_3$; most preferably R_9 is $-COCH_3$; and

Z is a steroid group;

or a pharmaceutically acceptable salt, ester or tautomeric form or derivative thereof.

Preferably the compound of the formula I is a compound of the formula III:



III

wherein:

R_4 is C_{1-6} alkyl, C_{1-6} hydroxyalkyl or C_{1-6}-alkoxy-C_{1-6}-alkyl; preferably C_{1-6} alkyl or C_{1-6} hydroxyalkyl more preferably $-CH_2OH$ or $-CH_3$; most preferably –

CH_2OH;

R_5 is C_{1-6} alkyl, C_{1-6} hydroxyalkyl or C_{1-6}-alkoxy-C_{1-6}-alkyl; preferably R_5 is C_{1-6} alkyl or C_{1-6} hydroxyalkyl; more preferably R_5 is $-CH_3$, $-C_2H_5$, $-CH_2OH$ or $-C_2H_4OH$; most preferably R_5 is $-CH_3$; and

R_7 is C_{2-6} alkyl, C_{1-6} hydroxyalkyl or C_{1-6}-alkoxy-C_{1-6}-alkyl; preferably R_7 is C_{1-6} hydroxyalkyl or C_{1-6}-alkoxy-C_{1-6}-alkyl; more preferably R_7 is-CH_2OH or C_{1-6} alkoxymethyl; most preferably R_7 is $-CH_2OH$.

More preferred are compounds of the formula III wherein:

R_4 is C_{1-6} hydroxyalkyl or C_{1-6} alkyl;

R_5 is C_{1-6} alkyl, C_{1-6} hydroxyalkyl; and

R_7 is C_{1-6} hydroxyalkyl or C_{1-6}-alkoxy-C_{1-6}-alkyl.

More preferred are compounds wherein:

R_4 is $-CH_2OH$ or $-CH_3$;

R_5 is $-CH_3$; and

R_7 is $-CH_3OH$.

Most preferred compounds of the formula III are compounds of the formula I wherein:

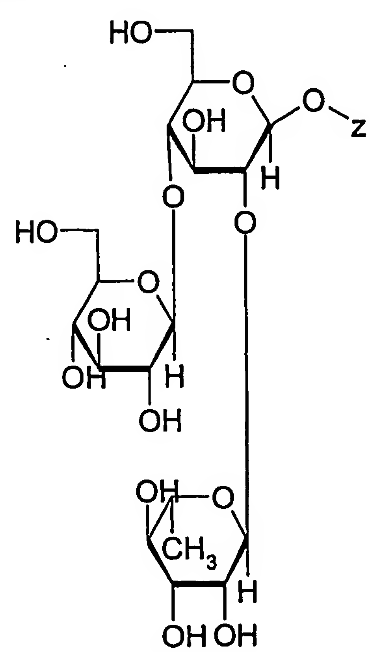
R_1 is rhamnose;

R_2 is $-OH$;

R_3 is glucose; and

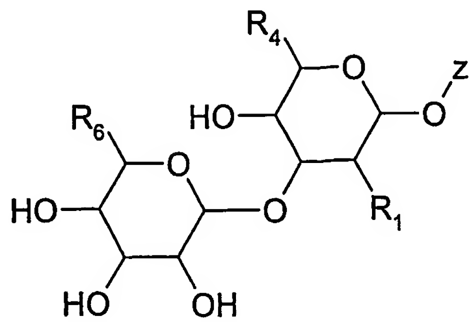
R_4 is $-CH_2OH$.

Most preferred are compounds of the formula I which are of the formula IV:



IV

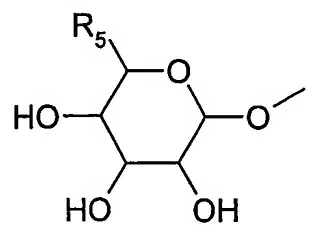
Also provided are compounds wherein the compound of the formula I is a compound of the formula V:



V

wherein:

R_1 is -OH, C_{1-6} alkoxy or NR_8R_9, or a monosaccharide of the formula IIa:



IIa

Preferably R_1 is -OH, or NR_8R_9; more preferably R_1 is NR_8R_9.

R_4 is C_{1-6} alkyl, C_{1-6} hydroxyalkyl or C_{1-6}-alkoxy-C_{1-6}-alkyl; preferably R_4 is C_{1-6} alkyl or C_{1-6} hydroxyalkyl more preferably R_4 is C_{1-6} alky; most preferably $-CH_3$;

R_5 is C_{1-6} alkyl, C_{1-6} hydroxyalkyl or C_{1-6}-alkoxy-C_{1-6}-alkyl; preferably R_5 is C_{1-6} alkyl or C_{1-6} hydroxyalkyl; more preferably R_5 is $-CH_3$ or $-CH_2OH$; most preferably R_5 is $-CH_3$; and

R_6 is C_{1-6} alkyl, C_{1-6} hydroxyalkyl or C_{1-6}-alkoxy-C_{1-6}-alkyl; preferably R_6 is C_{1-6} alkyl or C_{1-6} hydroxyalkyl more preferably R_6 is $-CH_2OH$ or $-CH_3$; most preferably R_6 is $-CH_2OH$;

R_8 is H, C_{1-6} alkyl or C_{1-6} acyl; preferably R_8 is H or C_{1-6} alkyl; more preferably R_8 is H or CH_3; most preferably R_8 is H;

R_9 is H, C_{1-6} alkyl or C_{1-6} acyl; preferably R_9 is H or C_{1-6} acyl more preferably R_9 is H or $-COCH_3$; most preferably R_9 is $-COCH_3$; and

Z is a steroid group.

Preferred compounds of the formula V are compounds in which:

R_1 is -OH, C_{1-6} alkoxy or NR_8R_9;

R_4 is C_{1-6} alkyl or C_{1-6} hydroxyalkyl;

R_6 is C_{1-6} alkyl or C_{1-6} hydroxyalkyl;

R_8 is H, C_{1-6} alkyl or C_{1-6} acyl; and

R_9 is H, C_{1-6} alkyl or C_{1-6} acyl.

More preferred compounds of the formula IV are those in which:

R_1 is -NH-C_{1-6}-acyl;

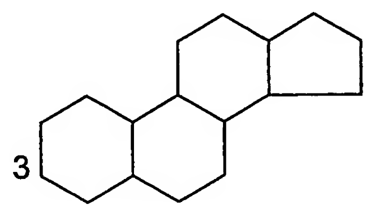
R_4 is C_{1-6} alkyl or -CH_2OH; and

R_6 is C_{1-6} hydroxyalkyl.

Most preferred are the compounds of the formula IV which are of the formula:

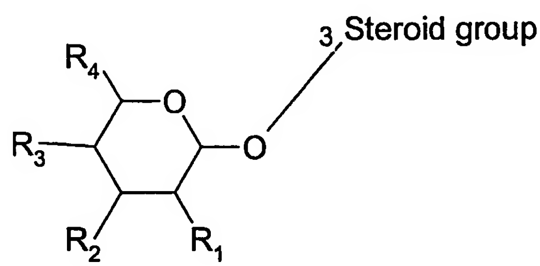
Galβ1→3(6-deoxy)GalNAcα-Z

The compounds of the formula I comprise a steroid group. The term "steroid group" means a group comprises the tetracyclic ring system shown as formula VI:



VI

Preferably the steroid group is attached to the rest of the molecule through the 3-position of the steroid group. For example compounds of the formula I above are preferably compounds of the formula:

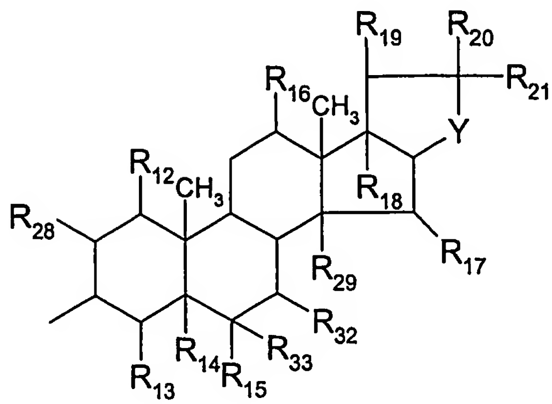


The steroid group may be cholestane, 5α-pregnane, androstane, estrane, cholesterol, cholane, a progestin, a glucocorticoid, a mineralocorticoid, an androgen such as dehydroepiandrosterone or its 7-keto analogue, a bile acid or other steroid. In one preferred embodiment the steroid core is a steroid that is in itself beneficial or neutral. By neutral is meant that the steroid itself has been passed suitable for use in a human or animal. By beneficial is meant that the steroid has effects of benefit to the human or animal if it were administered separately.

The steroid group may be a steroidal sapogenin derivable from plant sources or a steroidal sapogenin which is itself derivable from such plant steroidal sapogenins

by chemical modification.

In one embodiment the steroid group is a steroidal sapogenin of the formula VII:



VII

wherein:

R_{12} is H, OH, C_{1-6} alkyl or C_{1-6} alkoxy; preferably R_{12} is H or -OH; most preferably R_{12} is H;

R_{13} is H, -OH, =O, or C_{1-6} alkyl; preferably R_{13} is H or -OH; most preferably R_{13} is H;

R_{14} is H, -OH or C_{1-6} alkyl or R_{14} and R_{33} taken together represent the second bond of a double bond joining adjacent carbon atoms; preferably R_{14} is H or R_{14} and R_{33} taken together represent the second bond of a double bond joining adjacent carbon atoms;

R_{15} is H, or -OH, or R_{15} and R_{33} taken together are =O; preferably R_{15} is H, or R_{15} and R_{33} taken together are =O; more preferably R_{15} is H;

R_{16} is H, OH or =O; preferably R_{16} is H or =O; more preferably R_{16} is H;

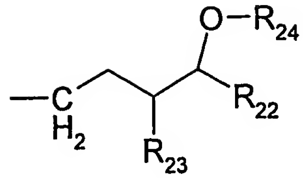
R_{17} is H, OH or =O; preferably R_{17} is H or-OH; more preferably R_{17} is H;

R_{18} is H, OH, C_{1-6} alkoxy or C_{1-6} alkyl; preferably R_{18} is H, OH, C_{1-6} alkoxy; more preferably R_{18} is H or OH; most preferably R_{18} is H;

R_{19} is H, OH, C_{1-6} alkyl or C_{1-6} alkoxy; preferably R_{19} is H, OH, C_{1-6} alkyl; more preferably R_{19} is H, OH or C_{1-6} alkyl; most preferably R_{19} is C_{1-6} alkyl; and particularly R_{19} is $-CH_3$;

R_{20} is H, OH, C_{1-6} alkoxy or C_{1-6} alkyl; preferably R_{20} is H, -OH, or C_{1-6} alkoxy; more preferably R_{20} is –OH or C_{1-6} alkoxy; most preferably R_{20} is –OH;

R_{21} is H, OH, C_{1-6} alkyl, C_{1-6} alkoxy or is a group of the formula VIII:



VIII

preferably R_{21} is a group of the formula VIII;

R_{22} is H, OH, C_{1-6} alkyl or C_{1-6} alkoxy; preferably R_{22} is H, OH, or C_{1-6} alkoxy; preferably R_{22} is H or OH, $-OCH_3$ or $-O-C_2H_5$; most preferably R_{22} is H;

R_{23} is H, OH, C_{1-6} alkyl, C_{1-6} hydroxyalkyl, C_{1-6}-alkoxy-C_{1-6}-alkyl, $=CH_2$ or $=CH-C_{1-6}$-alkyl; preferably R_{23} is C_{1-6} alkyl, C_{1-6} hydroxyalkyl, C_{1-6}-alkoxy-C_{1-6}-alkyl, $=CH_2$ or $=CH-C_{1-6}$-alkyl; more preferably R_{23} is C_{1-6} alkyl, C_{1-6} hydroxyalkyl or $=CH_2$; most preferably R_{23} is $-C_2H_4OH$, $-CH_2OH$, C_{1-6} alkyl, or $=CH_2$, even more preferably R_{23} is $-C_2H_4OH$, $-CH_2OH$, $-C_2H_5$, $-CH_3$ or $=CH_2$ and particularly R_{23} is $-CH_3$ or $=CH_2$; and

R_{24} is H, C_{1-6} alkyl, C_{1-6} acyl or a monosaccharide MS; preferably R_{24} is C_{1-6} alkyl, C_{1-6} acyl or a monosaccharide MS; more preferably R_{24} is C_{1-6} acyl or a monosaccharide MS; most preferably R_{24} is a monosaccharide MS.

R_{28} and R_{29} are the same or different and are H or OH; preferably R_{28} is H and R_{29} is $-OH$; more preferably both R_{28} and R_{29} are H;

R_{32} is H, OH or =O; preferably R_{32} is H or OH; most preferably R_{32} is H; and

R_{33} is H, or R_{33} and R_{15} taken together are =O, or R33 and R14 taken together represent the second bond of a double bond joining adjacent carbon atoms; preferably R_{33} is H or R_{33} and R_{14} taken together represent the second bond of a double bond joining adjacent carbon atoms;

MS is selected from a group consisting of arabinose, xylose, lyxose, ribose, glucose, mannose, galactose, allose, altrose, gulose, idose, talose, ribulose, xylulose, fructose, sorbose, tagatose, psicose, sedoheptulose, deoxyribose, fucose, rhamnose, 2-deoxy-glucose, quinovose, abequose, glucosamine, mannosamine, galactosamine, neuraminic acid, muramic acid, N-acetyl-glucosamine, N-acetyl-mannosamine, N-acetyl-galactosamine, N-acetylneuraminic acid, N-acetylmuramic acid, O-acetylneuraminic acid, N-glycolylneuraminic acid, fructuronic acid, tagaturonic acid, glucuronic acid, mannuronic acid, galacturonic acid, iduronic acid, sialic acid and guluronic acid; preferably MS is selected from a group consisting of glucose, galactose, mannose, fucose, N-acetyl-glucosamine, N-acetyl-galactosamine and sialic acid; most preferably MS is glucose; and

Y is N or O; preferably Y is O.

Preferred steroidal sapogenins of the formula VII are those in which R_{21} is of the formula VIII and Y is O.

More preferred steroidal sapogenins of the formula VII are those in which:

R_{12} is H, -OH

R_{13} is H or -OH;

R_{14} is H, or -OH or R_{14} and R_{33} taken together represent the second bond of a double bond joining adjacent carbon atoms;

R_{15} is H, or R_{15} and R_{33} taken together are =O;

R_{18} is H, -OH or C_{1-6} alkoxy

R_{19} is C_{1-6} alkyl;

R_{20} is H, -OH or C_{1-6} alkoxy;

R_{28} is H;

R_{32} is H, -OH or =O; and

R_{33} is H, or R_{33} and R_{15} taken together are =O, or R_{33} and R_{14} taken together represent the second bond of a double bond joining adjacent carbon atoms.

Most preferred are steroidal sapogenins of the formula VII in which:

R_{12}, R_{13}, R_{15} and R_{28} each represent H;

R_{14} is H, or R_{14} and R_{33} taken together represent the second bond of a double bond joining adjacent carbon atoms;

R_{16} is H, or =O;

R_{17} is H or -OH;

R_{18} is H or -OH;

R_{19} is H, or C_{1-6} alkyl;

R_{21} is of the formula VIII;

R_{22} is H, -OH, or C_{1-6} alkoxy;

R_{24} is C_{1-6} alkyl, C_{1-6} acyl, or glucose;

R_{29} is H or –OH; and

R_{32} is H or –OH.

The most preferred steroidal sapogenins of the formula VII are those in which

R_{12}, R_{13}, R_{15}, R_{16}, R_{17}, R_{22}, R_{28} each represent H;

R_{14} is H, or R_{14} and R_{33} taken together represent the second bond of a double bond joining adjacent carbon atoms;

R_{20} is -OH or C_{1-6} alkoxy;

R_{21} is of the formula VIII;

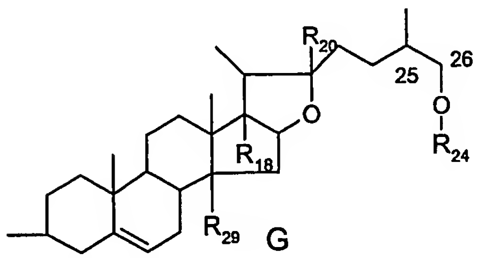
R_{23} is $-CH_3$ or $=CH_2$;

R_{24} is C_{1-6} acyl or glucose;

R_{29} is H or –OH; and

R_{32} is H.

The most preferred steroidal sapogenins of the formula VII are selected from the group consisting of: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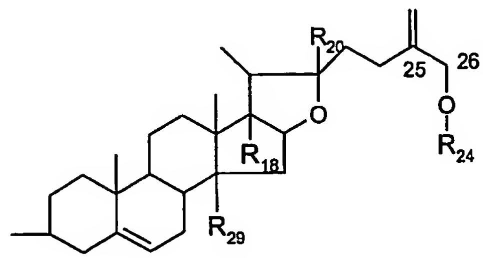
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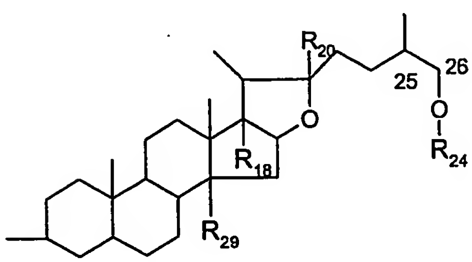
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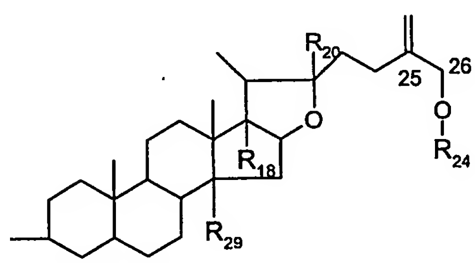

J

wherein:

R_{18} is H or OH;

R_{20} is OH or C_{1-6} alkoxy;

R_{24} is glucose or C_{1-6} acyl; and

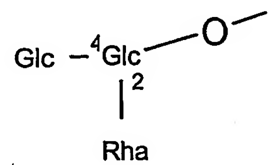
R_{29} is H or OH.

Particularly preferred compounds of the formula I in which the steroid group is of the formula VII are trigoneoside IVa, glycoside F, shatavarin I, compound 3, pardarinoside C, whose structures are summarised in Table 1.

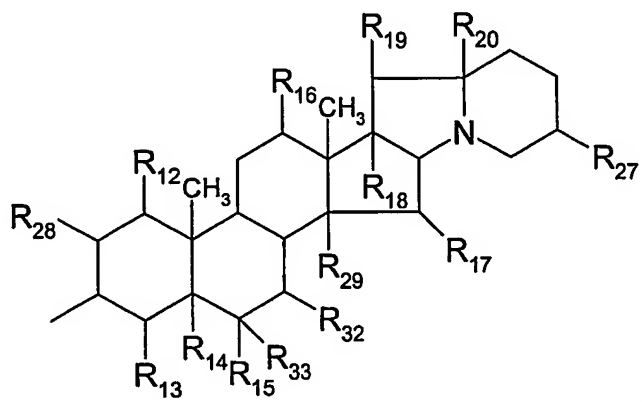
Table 1: Structural details of trigoneoside IVa, glycoside F, shatavarin I, compound 3 and pardarinoside C

Compound	Ref.	Steroid group	R_{18}	R_{20}	R_{29}	C^{25}	*R/S*	C^{26}
Trigoneoside IVa	55	G	H	-OH	H	$-CH_3$	*S*	Glc
Glycoside F	55	G	H	-OH	H	$-CH_3$	*R*	Glc
Shatavarin I	56	I	H	-OH	H	$-CH_3$	*S*	Glc
Compound 3	This document	H	H	-OH	H	$=CH_2$	?	Glc
Pardarinoside C	57	I	OH	-OMe	-OH	$-CH_3$	*R*	acetyl

In each case the saccharide group bonded to the steroid group at the 3-position is:



Alternatively the steroid group may be a steroidal sapogenin of the formula VIII:



IX

wherein:

R_{12} is H, -OH, C_{1-6} alkyl or C_{1-6} alkoxy; preferably R_{12} is H or -OH; most preferably R_{12} is H;

R_{13} is H, -OH, =O, or C_{1-6} alkyl; preferably R_{13} is H or -OH; most preferably R_{13} is H;

R_{14} is H -OH or C_{1-6} alkyl or R_{14} and R_{33} taken together represent the second bond of a double bond joining adjacent carbon atoms; preferably R_{14} is H or R_{14} and R_{33} taken together represent the second bond of a double bond joining adjacent carbon atoms;

R_{15} is H, or -OH, or R_{15} and R_{33} taken together are =O; preferably R_{15} is H, or R_{15} and R_{33} taken together are =O; more preferably R_{15} is H;

R_{16} is H, -OH or =O; preferably R_{16} is H or =O; more preferably R_{16} is H;

R_{17} is H, -OH or =O; preferably R_{17} is H or -OH; more preferably R_{17} is H;

R_{18} is H, -OH, C_{1-6} alkoxy or C_{1-6} alkyl; preferably R_{18} is H, -OH, C_{1-6} alkoxy; more preferably R_{18} is H or OH; most preferably R_{18} is H;

R_{19} is H, -OH, C_{1-6} alkyl or C_{1-6} alkoxy; preferably R_{19} is H, OH, or C_{1-6} alkyl; more preferably R_{19} is C_{1-6} alkyl; and particularly R_{19} is $-CH_3$;

R_{20} is H, -OH, C_{1-6} alkoxy or C_{1-6} alkyl; preferably R_{20} is H, -OH, or C_{1-6} alkoxy; more preferably R_{20} is –OH or C_{1-6} alkoxy; most preferably R_{20} is –OH;

R_{27} is H, -OH, C_{1-6} alkyl, C_{1-6} alkoxy or C_{1-6} hydroxyalkyl; preferably R_{27} is H, C_{1-6} alkyl or C_{1-6} alkoxy; more preferably R_{27} is H or C_{1-6} alkyl; most preferably R_{27} is methyl, ethyl or propyl;

R_{28} and R_{29} are the same or different and are H or -OH; preferably both R_{28} and R_{29} are H;

R_{32} is H, -OH or =O; preferably R_{32} is H or -OH; most preferably R_{32} is H; and

R_{33} is H, or R_{33} and R_{15} taken together are =O, or R_{33} and R_{14} taken together represent the second bond of a double bond joining adjacent carbon atoms; preferably R_{33} is H or R_{33} and R_{14} taken together represent the second bond of a double bond joining adjacent carbon atoms.

Preferred steroidal sapogenins of the formula IX are those in which:

R_{12} is H or –OH

R_{13} is H or –OH;

R_{14} is H or -OH, or R_{14} and R_{33} taken together represent the second bond of a double bond joining adjacent carbon atoms;

R_{15} is H or –OH

R_{16} is H, -OH or =O;

R_{17} is H, -OH or =O;

R_{18} is H or –OH

R_{27} is C_{1-6} alkyl; and

R_{28} and R_{29} are the same or different and each represent H or -OH;

R_{32} is H, -OH or =O.

More preferably steroidal sapogenins of the formula IX are those in which:

R_{12} is H or –OH

R_{13} is H or –OH;

R_{14} is H or -OH, or R_{14} and R_{33} taken together represent the second bond of a double bond joining adjacent carbon atoms;

R_{15} is H or –OH

R_{16} is H or =O;

R_{17} is H, -OH;

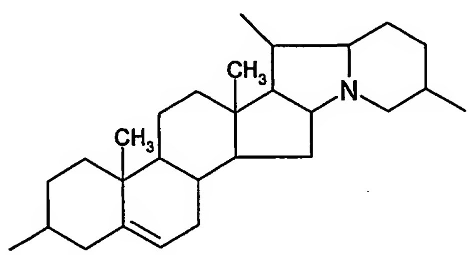
R_{18} is H or –OH;

R_{27} is C_{1-6} alkyl;

R_{28} and R_{29} are the same or different and each represent H or -OH; and

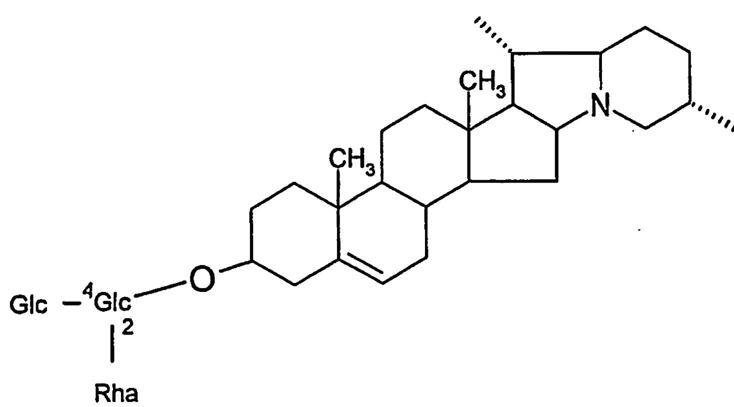
R_{32} is H or -OH.

More preferably steroidal sapogenins of the formula IX are those in of the general formula IXa:



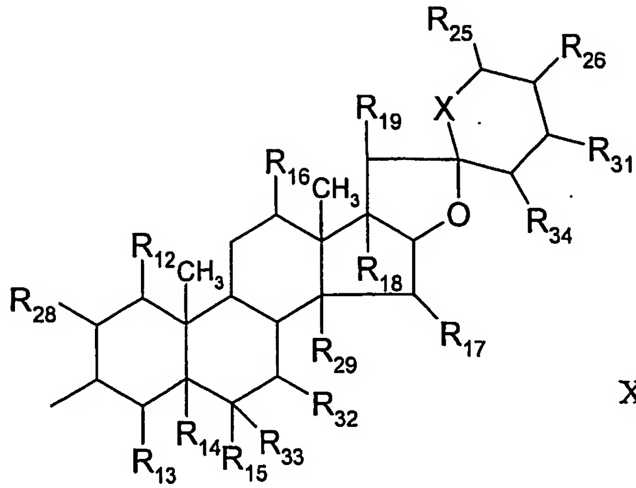
IXa

The most preferred compound of the formula I in which the steroid group is of the formula IX is:



isolatable from *Lilium macklineae* (59).

A further preferred group of steroidal sapogenins are those in which the steroidal sapogenin is of the formula XI:



XI

wherein:

R_{12} is H, OH, C_{1-6} alkyl or C_{1-6} alkoxy; preferably R_{12} is H or -OH; most preferably R_{12} is H;-

R_{13} is H, -OH, =O, or C_{1-6} alkyl; preferably R_{13} is H or -OH; most preferably R_{13} is H; -

R_{14} is H, -OH or C_{1-6} alkyl or R_{14} and R_{33} taken together represent the second bond of a double bond joining adjacent carbon atoms; preferably R_{14} is H or R_{14} and R_{33} taken together represent the second bond of a double bond joining adjacent carbon atoms; -

R_{15} is H, or -OH, or R_{15} and R_{33} taken together are =O; preferably R_{15} is H, or R_{15} and R_{33} taken together are =O; more preferably R_{15} is H; -

R_{16} is H, -OH or =O; preferably R_{16} is H or =O; more preferably R_{16} is H;

R_{17} is H, -OH or =O; preferably R_{17} is H or -OH; more preferably R_{17} is H;

R_{18} is H, -OH, C_{1-6} alkoxy or C_{1-6} alkyl; preferably R_{18} is H, OH, C_{1-6} alkoxy; more preferably R_{18} is H or -OH; most preferably R_{18} is H;

R_{19} is H, -OH, C_{1-6} alkyl or C_{1-6} alkoxy; preferably R_{19} is H, -OH, C_{1-6} alkyl; more preferably R_{19} is H, -OH or C_{1-6} alkyl; most preferably R_{19} is C_{1-6} alkyl; and particularly R_{19} is $-CH_3$;

R_{25} is H, -OH, C_{1-6} alkyl or C_{1-6} alkoxy; preferably R_{25} is H or -OH; more preferably R_{25} is H;

R_{26} is H, -OH, C_{1-6} alkyl, C_{1-6} hydroxyalkyl, C_{1-6}-alkoxy-C_{1-6}-alkyl, $=CH_2$ or $=CH-C_{1-6}$-alkyl; preferably R_{26} is C_{1-6} alkyl, C_{1-6} hydroxyalkyl, C_{1-6}-alkoxy-C_{1-6}-alkyl, $=CH_2$ or $=CHC_{1-6}$ alkyl; more preferably R_{26} is C_{1-6} alkyl, C_{1-6} hydroxyalkyl or $=CH_2$; most preferably R_{26} is $-C_2H_4OH$, $-CH_2OH$, C_{1-6} alkyl, or $=CH_2$, even more

preferably R_{26} is $-C_2H_4OH$, $-CH_2OH$, $-C_2H_5$, $-CH_3$ or $=CH_2$ and particularly R_{26} is $-CH_3$ or $=CH_2$;

R_{28} and R_{29} are the same or different and are H or -OH; preferably both R_{28} and R_{29} are H;

R_{31} is H or -OH; preferably R_{31} is H;

R_{32} is H, -OH or =O; preferably R_{32} is H or -OH; most preferably R_{32} is H;

R_{33} is H, or R_{33} and R_{15} taken together are =O, or R_{33} and R_{14} taken together represent the second bond of a double bond joining adjacent carbon atoms; preferably R_{33} is H or R_{33} and R_{14} taken together represent the second bond of a double bond joining adjacent carbon atoms;

R_{34} is H or -OH; preferably R_{34} is H; and

X is O, S or NH; preferably X is O or NH; more preferably X is O.

Preferred steroidal sapogenins of the formula XI are those in which:

R_{12} is H or –OH;

R_{13} is H or –OH;

R_{14} is H or -OH, or R_{14} and R_{33} taken together represent the second bond of a double bond joining adjacent carbon atoms;

R_{15}, R_{18} R_{28} and R_{29} are the same or different and each represent H or -OH,

R_{16} is H, OH or =O;

R_{17} is H, -OH or =O;

R_{18} is H, -OH or C_{1-6}-alkoxy;

R_{19} is H, or C_{1-6} alkyl;

R_{26} is H, C_{1-6} alkyl, C_{1-6} hydroxyalkyl, C_{1-6}-alkoxy-C_{1-6}-alkyl, $=CH_2$ or =CH-C_{1-6}-alkyl;

R_{29} is H or -OH;

R_{31} is H or -OH;

R_{32} is H, -OH or =O; and

R_{33} is H, or R_{33} and R_{15} taken together are =O, or R_{33} and R_{14} taken together represent the second bond of a double bond joining adjacent carbon atoms; and

R_{34} is H or -OH.

More preferred steroidal sapogenins of the formula XI are those in which:

R_{12}, R_{13}, R_{15} and R_{28} each represent H;

R_{14} is H, or R_{14} and R_{33} taken together represent the second bond of a double bond joining adjacent carbon atoms;

R_{16} is H, or =O;

R_{17} is H or -OH;

R_{18} is H or -OH;

R_{19} is H, or C_{1-6} alkyl;

R_{26} is C_{1-6} alkyl, C_{1-6} hydroxyalkyl or $=CH_2$;

R_{28} is H;

R_{29} is H or –OH;

R_{32} is H or –OH; and

R_{33} is H, or R_{33} and R_{14} taken together represent the second bond of a double bond joining adjacent carbon atoms.

Most preferred steroidal sapogenins of the formula XI are those in which:

R_{12}, R_{13}, R_{15}, R_{16}, R_{17}, R_{25}, R_{28}, R_{31}, R_{32} and R_{34}, each represent H;

R_{14} is H, or R_{14} and R_{33} taken together represent the second bond of a double bond joining adjacent carbon atoms;

R_{18} is H or –OH;

R_{19} is C_{1-6} alkyl;

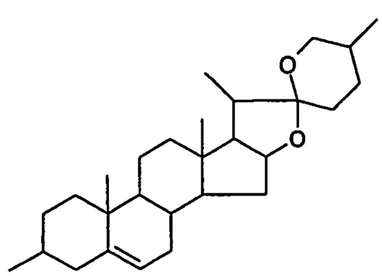
R_{26} is C_{1-6} alkyl or $=CH_2$;

R_{29} is H or –OH;

R_{32} is H;

R_{33} is H, or R_{33} and R_{14} taken together represent the second bond of a double bond joining adjacent carbon atoms.

The most preferred steroidal sapogenins of the formula XI are those selected from the groups:



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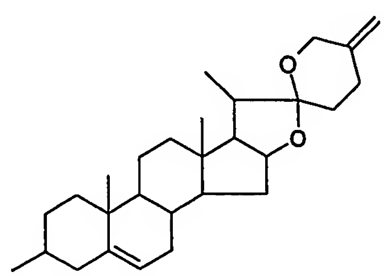

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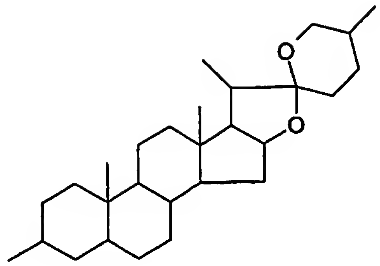

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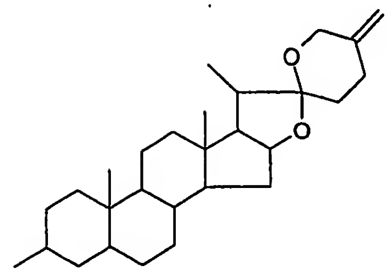

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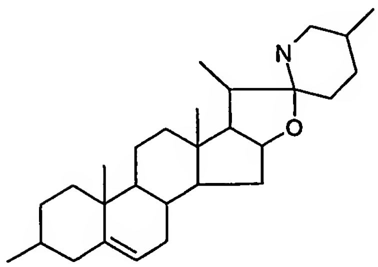

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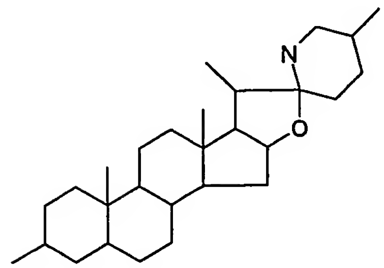


F

Particularly preferred steroidal sapogenins of the formula XI are diosgenin, yamogenin, tigogenin, neotigogenin, sarsasapogenin, smilagenin, hecogenin, solasodine or tomatidine.

Particularly preferred compounds of the formula I in which the steroidal group is of the formula XI are:

Shatavarin IV, (25R)shatavarin IV, deltonin, balanitin VI, compound 12 of Mimaki and Sahida (58).

Shatavarin IV is sarsasapogenin 3-O-α-L-rhamnopyranosyl-(1→2)-O-[β-D-glucopyranosyl-(1→4)]-β-D-glucopyranoside

Compound 12 is solasodine 3-O-α-L-rhamnopyranosyl-(1→2)-O-[β-D-glucopyranosyl-(1→4)]-β-D-glucopyranoside

Deltonin is (3β,25R)-spirost-5-en-3-yl-O-α-L-rhamnopyranosyl-(1→2)-O-[β-D-glucopyranosyl-β-D-Glucopyranoside.

Balanitin VI is (3β,25S)-spirost-5-en-3-yl-O-α-L-rhamnopyranosyl-(1→2)-O-[β-D-glucopyranosyl-β-D-Glucopyranoside.

Particularly preferred compounds of the formula I are those combining preferred steroid groups with preferred saccharide groups.

In a second aspect of the invention is provided the use of the compounds of the formula I in the manufacture of a medicament for the treatment of conditions

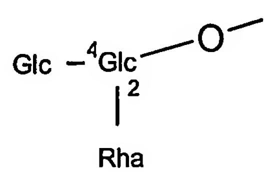
associated with raised activity of the enzyme core 2 GlcNAc-T. Examples of such conditions are described herein in the first aspect of the invention.

In a third aspect of the invention is provided pharmaceutical compositions comprising the compounds of the formula I.

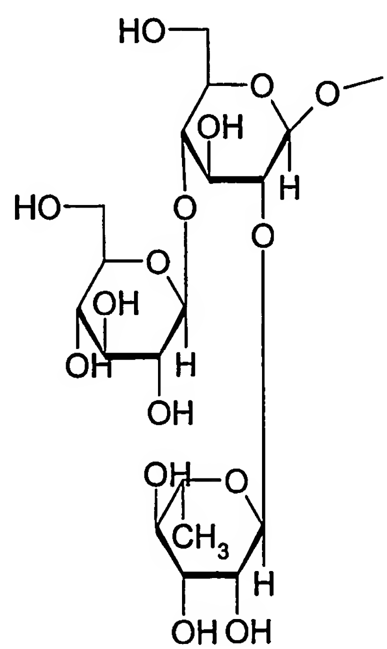
As used herein the term core 2 GlcNAc-T inhibitor means and inhibitor of the enzyme core 2-GlcNAc-T and preferably the ability of preparations comprising a core 2 GlcNAc-T enzyme activity described herein to incorporate UDP-6 ['H]-N-acetylglucosamine into products as measured in the assays described herein.

As used herein the term aglycone refers to compounds of the formula I wherein the saccharide moieties are not present. The compounds may have other substituents at the position occupied by the saccharide moiety. Particularly aglycones that are furostanol saponins when glycosylated may be in the ring closed state as the equivalent spirostanol saponins.

The shorthand annotation:



used in structures herein is used to denote the structure:



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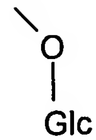
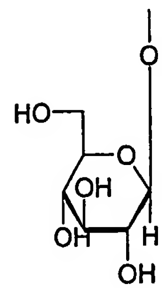


used in structures herein denotes the structure:



As used herein the shorthand annotation Glc is glucose and Rha is rhamnose.

For the avoidance of doubt the term C_{1-6} acyl is $–CO-C_{1-5}$-alkyl.

Brief Description of the Drawings

Figure 1 is a schematic flow chart illustrating the biosynthesis of O-glycan core structures.

Figure 2a is a graph illustrating that the activity of the enzyme core 2 GlcNAc-T can be induced by glucose. Human leukocytes (U937) were exposed to normal (5.8 mM) and high glucose (15 mM) for 24 hours at 37°C. Then the cells were lysed and the activity of core 2 GlcNAc-T measured. The data is presented as the means ± s.e.m., n = 28, the asterisk representing a significant difference ($P < 0.05$).

Figure 2b is a graph illustrating that crude extract F1 prepared from fenugreek seeds inhibits glucose-induced core 2 GlcNAc-T activity. Human leukocytes (U937) were exposed to normal (N, 5.8 mM; n = 3) and high glucose (G, I5 mM; n = 3) in the presence of fenugreek extract (1:1000 dilution; N-F, G-F). After 24 hours incubation, the activity of core 2 GlcNAc-T was determined in leukocyte cell lysates. The activity of core 2 GlcNAc-T is presented as pmoles/h/mg protein.

Figure 2c is a graph illustrating that crude extract F1 prepared from fenugreek seeds inhibits adherence of human leukocytes (U937) to cultured retinal capillary endothelial cells. After exposure to elevated glucose (15 mM) the level of leukocyte-endothelial cell adhesion was determined by labelling the leukocytes with carboxyfluorescein. The data is presented as the mean ± s.e.m., n = 3, the asterisk representing a significant difference ($P < 0.05$).

Figure 3 is a graph illustrating that crude extract F1 prepared from fenugreek seeds inhibits core 2 GlcNAc-T activity. Human leukocytes (U937) were exposed to 15 mM glucose for 24 hours at 37°C and the activity of core 2 GlcNAc-T was measured in leukocyte cell lysate in the presence of crude fenugreek seed extract (G-F1; 1:1000 dilution). The level of core 2 GlcNAc-T activity was measured by determining the formation of core 2 oligosaccharide (attachment of β1,6-linked GlcNAc to the Galβ1,3GlcNAc-acceptor). The data is presented as mean ± s.e.m. of three separate experiments.

Figure 4 is a schematic flow chart illustrating the extraction of fenugreek seeds and the subsequent purification of the fenugreek seed extract.

Figure 5 is a graph illustrating the inhibitory effect of crude fenugreek seed extract F1 and sub-fraction F2 purified from crude extract F1 on glucose-induced activity of core 2 GlcNAc-T in human leukocytes (U937). Cells were exposed to elevated glucose (15 mM) in the presence and absence of sub-fractions F1 and F2. After 24 hours incubation, the core 2 GlcNAc-T activity was determined in leukocyte cell lysates. The data represents the mean of two separate experiments.

Figures 6a and 6b are graphs illustrating the inhibitory effect of sub-fractions F8-F15 purified from crude extract F1 by silica-gel flash chromatography (Biotage) on glucose induced activity of core 2 GlcNAc-T in human leukocytes (U937). Cells were exposed to elevated glucose (G, 15 mM) in the presence of the sub-fractions. After 24 hours incubation, the core 2 GlcNAc-T activity was determined in leukocyte cell lysates. The data is presented as the mean ± s.e.m., n = 3, the asterisk representing a significant difference ($P < 0.05$).

Figure 7 is a graph illustrating that the aqueous phase of sub-fraction F13 inhibits glucose induced activity of core 2 GlcNAc-T in human leukocytes (U937). Sub-fractions F9 and F13 were thoroughly mixed with dichloromethane and the aqueous phase was filter sterilised and used in the cell-based assay for core 2 GlcNAc-T activity. Human leukocytes were exposed to elevated D-glucose (15 mM) in the presence and absence of the aqueous phases of sub-fractions F9 and F13. The results are presented as the mean of two separate experiments.

Figure 8 is a graph illustrating the inhibitory effect on glucose-induced activity of core 2 GlcNAc-T of sub-fractions purified from the aqueous phase of sub-fraction F 13 by HPLC with retention times F18.7-F41.1. Human leukocytes (U937)

were exposed to elevated D-glucose (15 mM) in the presence and absence of the HPLC sub-fractions with retention times F18.7-F41.1. The data presented is from one experiment. Sub-fractions G20.24, G20.69, G22.2, G39.9 and G41.1 (represented without a column in Figure 8) were not tested for their inhibitory effect on glucose-induced activity of core 2 GlcNAc-T.

Figure 9 is a graph illustrating the inhibitory effect of HPLC sub-fractions with retention times F19.13 and F19.37. Human leukocytes (U937) were exposed to elevated D-glucose (15 mM) for 24 hours in the presence and absence of the sub-fractions with retention times F19.13 and F19.37 (1: 1000 dilution). The data is presented as the mean ± s.e.m., n =3, the asterisk representing a significant difference ($P < 0.05$).

Figure 10 is a graph illustrating the inhibitory effect on glucose-induced activity of core 2 GlcNAc-T of sub-fractions purified from the aqueous phase of sub-fraction F13 by HPLC with retention times F20.01, F20.29 and F20.55. Human leukocytes (U937) were exposed to elevated D-glucose (15 mM) in the presence and absence of the sub-fractions with retention times F20.01, F20.29 and F20.55 and the activity of core 2 GlcNAc-T was measured after 24 hours. The data is the mean of two separate experiments.

Figure 11 is a graph illustrating that sub-fraction F20.55 inhibits core 2 GlcNAc-T in a cell-free assay. After exposing human leukocytes (U937) to 15 mM glucose for 24 hours at 37°C, the cells were lysed and then exposed to heated (H, 100°C) and non-heated (NH) sub-fraction F20.55 (1: 500 dilution). After 30 minutes exposure at 37°C, the activity of core 2 GlcNAc-T was measured. The level of core 2 GlcNAc-T activity was measured by determining the formation of core 2 oligosaccharide (attachment of β-1,6-linked GlcNAc to the Gal-1,3-GlcNAc-acceptor). The data is presented as mean ± s.e.m. of three separate experiments.

Figures 12a and 12b are graphs illustrating that elevated glucose increases core 2 GlcNAc-T activity in cultured bovine retinal vascular cells, namely capillary pericytes (Figure 13a) and capillary endothelial cells (Figure 13b). Near confluent cultures were exposed to normal glucose (N, 5.8 mM) and high glucose (G, 15 mM) for 24 hours at 37°C. The cells were lysed and the activity of core GlcNAc-T measured in cell lysates. The data is presented as the mean ± s.e.m. (n = 3-4), the asterisk representing a significant difference ($P < 0.05$).

Figures 13a and 13b are graphs illustrating that a crude extract F1 of fenugreek seeds prevents glucose-induced toxicity in cultured bovine retinal vascular cells, namely capillary pericytes (Figure 14a) and capillary endothelial cells (Figure 14b). Cells were exposed to normal (N, 5.8 mM) and high glucose (G, 25 mM) in the presence (N-F, G-F) and absence (N, G) of the fenugreek seed extract. After 4 days incubation, the number of viable cells was determined using a haemocytometer and trypan blue exclusion. The data is presented as the mean ± s.e.m., n = 18 separate experiments, the asterisk representing a significant difference ($P < 0.05$).

Figure 14 illustrates the structures of the five compounds isolated from fenugreek seeds.

Figure 15a and figure 15b are graphs illustrating the effect of purified trigoneoside IVa, glycoside F, and shatavarin IV on Core 2 GlcNAc-T activity in cell free (Figure 15a) and cell based (figure 15b) assays.

In cell free assays heart lysate from BB rats were incubated in the presence, and absence of 20 ng/ml of each compound. After 1h incubation at 37°C, the activity of core 2 GlcNAc-T was measured, and expressed as pmoles/h/mg protein. The results are the mean of 3-5 separate experiments.

In cell based assays human leukocytes (U937 cells) were exposed to 8 pg/ml human recombinant TNF-alpha in the presence and absence of 20 ng/ml of the test compound. After 24h incubation, the activity of core 2 GlcNAc-T was measured, and expressed as pmoles/h/mg protein.

The invention will now be described by reference to the following non limiting reference examples, figures and tables. Further embodiments falling within the scope of the claims will occur to those skilled in the art in the light of these.

Detailed Description of the Invention

Experimental methods

Compounds of the formula I can be extracted from a variety of plant species. Reference is made in this respect, and by way of example only, to Yoshikawa *et al* (55), Sasheda *et al* (59), Akhov *et al* (60), Joshi and Dev (61), Ravikumar *et al* (56), Vasil'eva and Paseshnichenko (62), Shimomura *et al* (57), Sharma and Sharma (63), Petit *et al* (64), Mimaki and Sashida (58), and Hostettman (65) and references therein). These documents are all incorporated herein by reference.

Alternatively, they can be synthesised by conventional organic chemistry

methods and techniques. Reference in this respect is made to carbohydrate and steroid chemistry textbooks such as "Essentials of Carbohydrate Chemistry and Biochemistry" by Thisbe K. Lindhorst (2000) Wiley, "Carbohydrates in Chemistry and Biology" edited by Beat Ernst, Gerald W. Hart and Pierre Sinay (2000) Wiley, "Essentials of Carbohydrate Chemistry" by John F. Robyt (1998) Springer Verlag, "Carbohydrate Chemistry" by Hassan S. El Khadem (1988), "Carbohydrate Building Blocks" by Mikael Bols (1996), “Glycochemistry: Principles, Synthesis, and Applications” edited by P.G. Wang and C.R. Bertozzi (2001) Marcel Dekker, N.Y. and "Carbohydrate Chemistry" by the Royal Society of Chemistry Staff (1989) CRC Press.

The compounds of the present invention can be prepared from commercially available aglycones or by isolation of the aglycone or other precursor either from fenugreek seeds or from another plant source and subsequent chemical modification of the precursor.

The skilled worker will for example be aware of many sources of spirostanol and furostanol aglycones such as diosgenin, yamogenin, tigogenin, neotigogenin, sarsapogenin, smilagenin, hecogenin, solasodine or tomatidine (for example Hostettman and references therein (65)),

Specifically for methods of synthesis of spirostanol saponins having 2, 4 branched oligosaccharide moieties, from diosgenin see Du *et al* 2003 (73). This reference also makes further reference to the synthesis if other glycosylated steroids, for example from cholesterol. The methods disclosed can be used to synthesize compounds in which steroids are chemically glycosylated to form compounds of the formula I.

Further reference is made to Li *et al* (66) for synthesis of a trisaccharide substituted spirostanol saponins, Deng *et al* (67), for synthesis of a variety of tri and tetra saccharide substituted spirostanol saponins, Li *et al* (68), Yu *et al* (69), Yu *et al* (70) for methods of synthesis of furostanol saponins and interconversion of spirostanol and furostanol saponins, Yu and Tao (71), Cheng *et al* (72) and Du *et al* (73). These references also provide information and further references on derivatisation of monosaccharide hydroxyalkyl groups.

Methods of synthesising Galβ1-3(6deoxy)GalNAcα– conjugates are given in Paulsen et al (48). These methods may be adapted by the skilled worker in

combination with other methods referenced herein to synthesize other compounds of the formula I.

Cell culture

Bovine retinal capillary endothelial cells (BREC) and pericytes (BRP) were established from bovine retinas dissected from eyes of freshly slaughtered cattle as described previously (48). Briefly, the isolated retinas were homogenised in serum-free minimal essential medium (MEM, Gibco, Paisley, UK) and filtered through 85 μm nylon mesh. The trapped microvessels were digested with collagenase-dispase (1 mg/ml) for 30 minutes (BRP) and 90 minutes (BREC) at 37°C and filtered through a 53 μm nylon mesh. For growth of endothelial cells (BREC), the digested microvessels were plated in gelatine coated tissue culture flasks and maintained in MEM supplemented with 10% pooled human serum, 2 mM glutamine, 100 IU/ml penicillin and 100 μg/ml streptomycin. For growth of pericytes (BRP), the microvessels were plated in tissue culture flasks in growth medium supplemented with 10% foetal calf serum. The cells were used at passage 2-3. The cells were characterised using morphological criteria and by immunostaining with an antibody against factor VIII related antigen and 3G5-pericyte marker.

The human leukocytic cell-line (U937) was cultured in RPMI supplemented with 10% foetal calf serum, 2 mM glutamine, 100 IU/ml penicillin and 100 μg/ml streptomycin.

Cell-based assay of core 2 GlcNAc-T activity

To investigate the potential of fenugreek to pharmacologically inhibit core 2 GlcNAc-T, enzyme activity was measured in leukocytes exposed to normal glucose (5.8 mM) and high glucose (15 mM) for 24 hours at 37°C. After incubation, the cells were lysed and frozen at -20°C until used for the measurement of core 2 GlcNAc-T. The activity of core 2 GlcNAc-T in cultured bovine retinal capillary pericytes (BRP) and endothelial cells (BREC) was also measured.

Cell-free assay of core 2 GlcNAc-T activity

Core 2 GlcNAc-T immobilised on Sepharose beads were used for this assay. For core 2 GlcNAc-T immunoprecipitation, as well as for Western blots, a polyclonal

antibody against core 2 GlcNAc-T was used. Cells were lysed on ice in the following lysis buffer: 20 mM Tris-HCL, pH 7.4/1% Triton X-100, 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 0.2 mM sodium vandate, 1 mM PMSF 1 μg/ml aprotinin, 10 μg/ml leupeptin. The lysate was incubated at 4°C for 20 minutes with constant agitation and insoluble material removed by centrifugation (14,000g for 5 minutes at 4°C). The clarified lysate was incubated with staphylococcal protein A-Sepharose CL-4B conjugated primary antibody for 2 hours with constant agitation at 4°C. The immunoprecipitates were washed with Tris buffered saline (10 mM Tris-HCL, pH 7.4, 150 mM NaCl) containing 0.5% Triton X-100 and used in the measurement of core 2 GlcNAc-T in the presence and absence of potential inhibitors.

Measurement of core 2 GlcNAc-T activity:

To measure core 2 GlcNAc-T activity, leukocytes were washed in PES, frozen and lysed in 0.9% Triton X-100 at 0°C. The activity of core 2 GlcNAc-T was measured as described previously (41). Briefly, the reaction was performed in a reaction mixture containing 50 mM 2(N-morpholino)ethanesulfonic acid (MES, Sigma, Dorset, UK), pH 7.0, 1 mM UDP-6 ['H]-N-acetylglucosamine (16,000 dpm/nmol, NEN Life Science Products, Hounslow, UK), 0.1 M GlcNAc (Sigma, Dorset, OK), 1 mM Galβ1-3GalNAcα-p-nitrophenol (Sigma, Dorset, UK) as substrate, and 16 μl of cell lysate (100-200 μg protein) for a final volume of 32 μl. After incubating the mixture for 1 hour at 37°C, the reaction was terminated with 1 ml of ice-cold distilled water and processed on a C18 Sep-Pak column (Waters-Millipore, Watford, UK). After washing the column with 20 ml of distilled water, the product was eluted with 5 ml of methanol. The radioactivity of the samples was counted in a liquid scintillation β-counter (LKB-Wallac, London, UK). Endogenous activity of core 2 GlcNAc-T was measured in the absence of the added acceptor. The specific activity was expressed as pmoles/h/mg of cell protein. In each case, the protein concentration was determined with BioRad protein assay (BioRad, Hertfordshire, UK).

Leukocyte-endothelial adhesion assay

Adhesion of leukocytes to endothelial cells was examined by labelling with carboxyfluorescein (Molecular Probe, UK). The assay is well established (41).

Briefly, endothelial cells were grown to a confluent state in order to provide an endothelial cell surface for the adhesion of the carboxyfluorescein-labelled leukocytes (U937). After treatment, the leukocytes were centrifuged (14 000 g for 1 minute) and washed twice with serum-free RPML The cells were then resuspended in 1 ml of serum-free RPMI containing 50 μg/ml carboxyfluorescein. The cells were counted with a haemocytometer and a known number added to the endothelial cells. After 30 minutes incubation at 37°C, non-adherent leukocytes were removed by washing with serum-free RPMI and the dishes fixed in 3.7% formalin in PBS. Attached leukocytes were counted in 10 random high-powered fields (x 100) by fluorescence microscopy. The results were expressed as percentage of adherent leukocytes/field.

Glucose toxicity

BRP and BREC were plated in 3 cm tissue culture dishes and incubated in growth medium for 24 hours at 37°C. Then the cells were incubated in fresh growth medium containing normal glucose (5.8 mM) or elevated glucose (25 mM) in the absence or presence of fenugreek sub-fractions. After 4 days incubation, the number of viable cells was counted using a haemocytometer and trypan blue and the results expressed as percentage of control (5.8 mM glucose). After treatment, some of the cells were stored for measurement of core 2 GlcNAc-T activity.

Biological activity of crude fenugreek seed extract

As shown in Figure 2a, 24 hour exposure to elevated D-glucose significantly increases the activity of core 2 GlcNAc-T in human leukocytes (U937). It has now been found that crude extract prepared from fenugreek seeds has the potential to inhibit glucose-induced activity of core 2 GlcNAc-T in human leukocytes (Figure 2b) and leukocyte-endothelial cell adhesion (Figure 2c). Leukocyte-endothelial cell adhesion was measured by adding a known number of leukocytes stained with carboxyfluorescein to a monolayer of retinal capillary endothelial cells. The number of attached leukocytes was then counted under a fluorescence microscope using 10-random fields.

The results illustrated in Figure 3 were obtained by exposing human leukocytes (U937) to elevated glucose for 24 hours. The cells were then lysed, incubated with crude fenugreek seed extract F1 and core 2 GlcNAc-T activity was

measured after 30 minutes incubation.

Preparation and purification of fenugreek seed extracts: example 1.

Fenugreek seed extracts were obtained as follows (see Figure 4). Fenugreek seeds (Indian fenugreek seeds obtained as Methi seeds from FUDCO, 184 Ealing Road, Wembley, Middlesex, UK) were ground in a hammer mill and filtered through nylon mesh. 820g of the dark-yellow powder obtained were defatted by continuous washing with hexane in a soxhlet apparatus for eight hours. Then the plant material was dried and continuously extracted for 8 hours with ethanol. Filtration to remove solid residues and concentration in vacuo of the ethanol yielded a semi-solid brown crude extract labelled F1 (65g). Since this appeared to contain residual oil, 50g of the crude extract F1 were shaken with cold hexane (500 ml). The hexane soluble material was filtered off and the solvent removed to give F3 (15.4 g), while the insoluble residue was collected on the filter paper and dried to give F2 (27 g).

Normal phase silica-gel flash chromatography was now employed using a commercial kit (Biotage). F2 (5g) was adsorbed onto silica-gel (5g) and packed into the sample barrel that was connected by short tubing to the main chromatography column (20 cm × 4 cm) containing silica-gel KP-Sil. The sample was eluted onto and through the column with a succession of solvents of increasing polarity consisting of varying mixtures of light petroleum (40/60), chloroform, methanol and acetone. Eluting sub-fractions were examined by TLC and similar ones pooled to give seven main eluted sub-fractions F8 to F14 representing compounds of increasing polarity. The silica was removed and shaken with 100% methanol, filtered and dried to give a residue labelled F15. Weights and approximate elution solvents for each sub-fraction are set out in Table 2.

Table 2: Separation of sub-fraction F2 into sub-fractions F8-F15 using flash chromatography

Sub-fraction	Weight	Eluent
F8	0.03 g	light petroleum (40/60) 100% to chloroform 100%
F9	0.10 g	chloroform:methanol 90: 1 0
F10	0.02 g	chloroform:methanol from 90: 10 to 80:20
F11	0.03 g	chloroform:methanol from 80:20 to 70:30
F12	0.82 g	chloroform:methanol from 70:30 to 60:40
F13	1.58 g	chloroform:methanol 50:50

Sub-fraction	Weight	Eluent
F14	0.01 g	chloroform:methanol:acetone 30:30:40 to acetone 100%
F15	0.14 g	eluted from silica-gel with methanol

Biological activity of purified fenugreek seed extracts

The potential of these purified sub-fractions to inhibit glucose-induced activity of core 2 GlcNAc-T in leukocytes was examined. Firstly, it was demonstrated that sub-fraction F2 can inhibit glucose-induced core 2 GlcNAc-T activity in leukocytes (Figure 5). Further experiments demonstrated the presence of the inhibitor of core 2 GlcNAc-T in sub fractions F13 and F14 (Figures 6a and 6b).

Sub-fractions F9 and F13 were then analysed. An aqueous aliquot (0.5 ml) of both subfractions F9 and F13 was extracted with 1 ml of dichloromethane, the aqueous phase was removed, filter-sterilised by filtration through 0,22μm filter and used in the cell-based assay for core 2 GlcNAc-T activity. Human leukocytes were exposed to elevated D-glucose (15 mM) in the presence and absence of the aqueous phases of sub-fractions F9 and F13, The results are presented in Figure 7 showing the presence of the core 2 GlcNAc-T inhibitor in the aqueous phase of sub-fraction F13.

The aqueous phase of sub-fraction F13 was purified by HPLC into sub-fractions F18.7-F41.1 coded by their HPLC retention times. The aqueous phase of sub-fraction F13 was directly injected onto the HPLC operating under reversed-phase conditions (Hewlett Packard 1050/\100 series), Separation was achieved with an octadecyl-bonded column with a methanol/water mobile phase, Components eluted from the column were detected by a UV detector operating at a fixed wavelength of 22 nm, These components were revealed as peaks on the chromatographic trace from the mass spectrometer detector. The sub-fractions thus obtained were concentrated *in vacuo* to dryness, re-dissolved in phosphate buffered saline (PBS) and filter-sterilised. Cell-based assays for core 2 GlcNAc-T activity were carried out and the results suggested the presence of core 2 GlcNAc-T inhibitor in sub-fractions F19-F20.03 (see Figures 8 and 9).

Subsequently larger amounts of the aqueous phase of sub-fraction F13 were purified similarly by HPLC operating under reversed-phase conditions on a phenyl-bonded column with a methanol/water mobile phase into sub-fractions with retention times of 20,01, 20.29 and 20.55, which are equivalent to sub-fractions F19.13, Fl9.37 and F19.44 above. Cell based assays for core 2 G1cNAc-T activity confirmed the

presence of the core 2 G1cNAc-T inhibitor in these sub-fractions F20,01, F20.29 and F20.55 (Figure 10a). The inhibition of core 2 GlcNAc-T by HPLC purified sub-fraction F20.55 has been demonstrated using the cell-free assay system (Figure 11). After exposing human leukocytes (U937) to 15 mM glucose for 24 hours at 37°C, the cells were lysed and then exposed to heated (H, 100°C) and non-heated (NH) sub-fraction F20.55 (1:500 dilution). After 30 minutes exposure at 37°C, the activity of core 2 GlcNAc-T was measured. As shown in Figure 11, it was found that sub-fraction F20.55 directly inhibits core 2 GlcNAc-T in a cell-free assay. Heating of sub-fraction F20.55 only slightly altered the level of core 2 GlcNAc-T inhibition.

Structural analysis of the core 2 GlcNAc-T inhibitor.

The core 2 GlcNAc-T inhibitor in the sub-fraction F20.55 has been identified through NMR analysis of a sample dissolved in CD_3OD. The following NMR experiments were performed: 1D proton, 2D DQF-COSY (^1H-^1H correlation) [8 hours], 2D edited HSQC (^1H-^{13}C one-bond correlation with multiplicity editing) [22 hours], 2D TOCSY (^1H-^1H relayed correlation) [2 × 8 hours].

^1H and ^{13}C NMR data for the core 2 GlcNAc-T inhibitor in sub-fraction F20.55 is presented in Tables 3 and 4.

Table 3: ^1H NMR data (sample in deuteriopyridine)

Sample	Assignment
0.90 singlet	18-H
1.03 doublet 1 6.7 Hz	27-H
1.06 singlet	19-H
1.33 doublet J 7. 1 Hz	21-H
1.77 doublet J 604 Hz	Sugar-Me
2.24 dq J 6.9 Hz	20-H
5.29 multiplet	6-H

Table 4: ^{13}C NMR data (sample in deuteriopyridine)

Aglycone portion

Sample	Assignment
37.5	1
30.1	2
78.0	3
38.9	4
140.7	5
121.8	6
32.3	7
31.6	8
50.3	9
37.2	10
21.1	11
39.9	12
40.7	13
56.5	14
32.5	15
81.1	16
63.8	17
16.4	18
19.4	19
40.7	20
16.4	21
10.6	22
110.6	23
37.1	24
28.3	25
34.4	26
75.3	27
17.4	28

Sugar portion

Sample	Assignment
100.2	Glc 1'
77.7	2'
76.3	3'
81.9	4'
77.7	5'
62.1	6'
102.0	Rha 1"
72.5	2"
72.7	3"
74.1	4"
69.5	5"
18.6	6"
105.1	Glc 1'''
75.1	2'''
78.4	3'''
71.6	4'''
78.2	5'''
61.6	6'''
105.1	Glc 1''''
75.2	2''''
78.6	3''''
71.6	4''''
78.4	5''''
62.8	6''''

The compound of interest was identified as Trigoneoside IVa, a known constituent of Fenugreek seeds (55)

Bulk preparation of trigoneoside IVa, protodioscin, compound 3 and glycoside F

Crushed seeds (360 g, product of Deep Foods, Inc., Union, NJ 07083, USA) were extracted successively with heptane (2 × 700 ml), acetone (4 × 600 ml) and MeOH (4 × 600 ml) by boiling under reflux for 2 hrs each. The extracts were filtered and evaporated to dryness under vacuum and analyzed by LC/MS for the presence of furostanol saponins previously reported from this plant (55, 74, 75). The methanol

extract (82 g, 22.7% (w/w) of the seeds) was found to contain the target compounds.

The initial extraction of the seeds with heptane and acetone removed most of the less polar materials and improved subsequent chromatography. Further de-fatting can be accomplished by partitioning the methanol extract between butanol and water. However, methanol extract contained relatively little polar material and an enriched saponin containing fraction can be obtained by a solid phase extraction using a styrenic resin such as Diaion HP20 (or SP207, HP20SS, SP207SS, all available from Sigma-Aldrich) resin without subjecting the extract to further de-fatting.

The MeOH extract (CDXA-13-132-1, 81.2 g) was dissolved in water-MeOH (6:4, 400 ml) and loaded onto a Diaion HP20 (Supelco Diaion HP 20, 350 g, 5.0 × 30 cm) and eluted with water-MeOH (4:6, 600 ml), MeOH (2 L), and acetone (2L). 250 ml fractions were collected. The fractions were analyzed by HPLC and those with similar compositions were combined to produce 7 pools (CDXA-13-133 F1 to F7). The pool CDXA-13-133-F5 (22.5g, 27.7% w/w of the extract) was found to contain the majority of the desired saponins.

This pool (22.0 g,) was chromatographed on normal phase silica (445 g, Merck silica gel 60, 70-230 mesh, 0.0763 to 0.200 mm, 5.0 × 30 cm) and eluted with 3 L each of dichloromethane-MeOH-water systems of following compositions: a) 80:20:3, b) 75:25:3, c) 70:30:3, and d) 65:35:3. 250 ml fractions were collected, analyzed by HPLC and combined into 11 pools (CDXA-13-137-F1 to F11).

The fractions F6 and F7 were combined, dried (10.0 g, 45 %) and chromatographed on C8 Silica (350 g, Phenomenex Luna C8(2), 5 micron, 100 A, 5.0 × 28 cm) and eluted with MeOH-water systems of following compositions: 4:6 (800 ml), b) 5:5 (2 L), c) 55:45 (5 L) 6:4 (1 L), d) 65:35 (1 L), e) 7:3 (1 L), f) 8:2 (1 L) and MeOH (1 L). The fractions were analyzed by HPLC and combined to give 29 pools (CDXA-13-138-F1 to F29). 250 ml fractions were collected.

Fractions F13 to F16 were dried (1.155 g, 11.6 %) and purified by reverse phase HPLC using a Gilson semi preparative HPLC system consisting of a UV/Vis detector model 155, pump model 321, and liquid handler model 215.

Chromatographic conditions:

Column: Phenomenex Luna C18(2), 5 micron, 150 × 21.2 mm

Mobile Phase: Acetonitrile-Water (28:72)

Sample size: 15 mg of each fraction per injection

Detection: UV 205 nm

Five peaks were collected, P1 to P5, (Figure ** 1 to 5) and were identified by comparison of ^{1}H, ^{13}C NMR and Mass spectral data with those reported in the literature for trigoneoside IVa, its 25 (S) isomer - glycoside F. A further similar compound, compound 3 was detected. This compound has not been previously described.

NMR spectra were recorded in d_5 Pyridine. The proton spectra were recorded on a Varian Inova VXRs -300 instrument at 300 MHz and the carbon spectra were recoded on a Varian Inova 400 instrument at 100 MHz.

Mass spectra were recorded on a Finnigan LCQ Deca instrument in APCI mode.

Peak 1, Trigoneoside IVa: White solid (90 mg, 0.025 % w/w of the seeds). ^{1}H NMR (pyridine-d5, 400 MHz, δ): 0.90 (3H, s, 18-H_3), 1.04 (3H, d, *J*=6.8 Hz, 27-H_3), 1.07 (3H, s, 19-H_3), 1.34 (3H, d, *J*=6.8 Hz, 21-H_3), 1.79 (3H, s, *J*=6.0 Hz, Rha-6''-H_3), 3.88 (1H, m, 3-H), 4.09 (2H, m, 16-H_2), 4.84 (1H, d, *J*=7.6 Hz, Glc-1''''-H), 4.97 (1H, overlapped, Glc-1'-H), 5.16 (1H, d, *J*=7.6 Hz, Glc-1'''-H), 5.29 (1H, d like, 6-H), 6.29 (1H, br s, Rha-1''-H).

Peak 2, Compound C / protodioscin: White solid (120 mg, 0.033%). ^{1}H NMR (pyridine-d5, 400 MHz, δ): 0.90 (3H, s, 18-H_3), 1.04 (3H, d, *J*=6.8 Hz, 27-H_3), 1.07 (3H, s, 19-H_3), 1.34 (3H, d, *J*=6.8 Hz, 21-H_3), 1.66 (3H, s, *J*=6.0 Hz, Rha-6'''-H_3), 1.79 (3H, s, *J*=6.0 Hz, Rha-6''-H_3), 3.88 (1H, m, 3-H), 4.09 (2H, m, 16-H_2), 4.84 (1H, d, *J*=8.0 Hz, Glc-1''''-H), 4.97 (1H, overlapped, Glc-1'-H), 5.90 (1H, br s, Rha-1'''-H), 5.32 1H, d like, 6-H), 6.45 (1H, br s , Rha-1''-H).

Peak 3, Compound 3: White solid (30 mg, 0.008%). ^{1}H NMR (pyridine-d5, 400 MHz, δ): 0.89 (3H, s, 18-H_3), 1.06 (3H, s, 19-H_3), 1.34 (3H, d, *J*=6.4 Hz, 21-H_3), 1.66 (3H, s, *J*=6.0 Hz, Rha-6'''-H_3), 1.79 (3H, s, *J*=6.0 Hz, Rha-6''-H_3), 3.88 (1H, m, 3-H), 4.84 (1H, d, *J*=8.0 Hz, Glc-1''''-H), 4.97 (1H, overlapped, Glc-1'-H), 5.32 1H, d like, 6-H), 5.90 (1H, br s, Rha-1'''-H), 6.45 (1H, br s, Rha-1''-H).

Peak 4, Glycoside F: White solid (120 mg, 0.033%). ^{1}H NMR (pyridine-d5, 400 MHz, δ): 0.90 (3H, s, 18-H_3), 1.00 (3H, d, J=6.4 Hz, 27-H_3), 1.06 (3H, s, 19-H_3), 1.35 (3H, d, J=6.4 Hz, 21-H_3), 1.79 (3H, s, J=6.0 Hz, Rha-6-H_3), 3.88 (1H, m, 3-H), 3.97 (2H, m, 16-H_2), 4.84 (1H, d, J=7.6 Hz, Glc-1''''-H), 4.97 (1H, overlapped, Glc-1'-H), 5.16 (1H, d, J=7.6 Hz, Glc-1'''-H), 5.29 (1H, d like, 6-H), 6.29 (1H, br s, Rha-1''-H).

Table 5. 13C NMR data of Peaks 1 to 5 (in pyridine-d5, 100 MHz)

	Peak				
Carbon	1	2	3	4	5
1	37.5	38	38	38	38
2	30.1	30.7	30.7	30.6	30.7
3	78.1	78.6	78.6	78.6	78.6
4	38.9	39.4	39.5	39.4	39.5
5	140.7	141.2	141.3	141.2	141.2
6	121.8	122.4	122.4	122.4	122.4
7	32.3	32.9	32.9	32.7	32.8
8	31.7	32.2	32.2	32.2	32.2
9	50.3	50.8	50.9	50.8	50.8
10	37.1	37.6	37.6	37.6	37.6
11	21.1	21.6	21.6	21.6	21.6
12	39.9	40.4	40.4	40.4	40.4
13	40.8	41.3	41.3	41.3	41.3
14	56.6	57.1	57.1	57.1	57.1
15	32.5	33	33	32.8	33
16	81.1	81.6	81.6	81.6	81.6
17	63.8	64.3	64.3	64.3	64.3
18	16.5	17	17	17	17
19	19.4	19.9	20	19.9	19.9
20	40.7	41.2	41.2	41.2	41.2
21	16.5	17	17	17	17
22	110.7	111.2	111.2	111.2	111.2
23	37.1	37.6	37.7	37.7	37.7
24	28.3	28.8	28.9	28.9	28.9
25	34.4	34.9	35	34.8	34.8
26	75.4	75.9	75.9	75.8	75.8
27	17.4	18	18	18	18
G1'	100	100.5	100.8	100.5	100.8
G2'	77.3	77.8	78.5	77.7	78.4
G3'	76.2	76.7	78.3	76.6	78.2

Carbon	Peak				
	1	2	3	4	5
G4'	81.9	82.5	78.8	82.5	78.9
G5'	77.7	78.2	77.4	78.2	77.4
G6'	62.1	62.5	61.8	62.5	61.7
rha1"	101.8	102.3	102.6	102.3	102.5
rha2"	72.4	73	73.1	73	73
rha3"	72.7	73.3	73.3	73.3	73.3
rha4"	74.1	74.6	74.6	74.6	74.6
rha5"	69.5	70	70.1	70	70
rha6"	18.7	19.2	19.2	19.2	19.2
glc1/rha1'''	105.2	105.7	103.4	105.7	103.4
glc2/rha2'''	75	75.5	73.1	75.5	73
glc3/rha3'''	78.4	79	73.2	79	73.2
glc4/rha4'''	71.2	71.7	74.4	71.7	74.4
glc5/rha5'''	78.2	78.7	70.9	78.8	70.9
glc6/rha6'''	61.8	62.3	19	62.3	19
26-*O*-G1''''	105.1	105.7	105.7	105.4	105.4
G2''''	75.2	75.7	75.7	75.7	75.7
G3''''	78.6	79.1	79	79.1	79.1
G4''''	71.6	72.1	72.1	72.1	72.1
G5''''	78.4	79	79	79	79
G6''''	62.8	63.3	63.3	63.3	63.3

Table 6. Summary

Compound ID	Name	Yield (mg)
F1	Trigoneoside IVa	90 mg
F2	Compound C / Protodioscin	120 mg
F3	Compound 3	30 mg
F4	Glycoside F	120 mg
F5	Trigonelloside C	300 mg

Chemical structures for the five compounds are given in figure 15.

Other compounds

Shatavarin IV (figure 15) isolated from *Asparagus racemosus* (56), and protodioscin from *Tribulus terrestris* (but also isolatable from fenugreek as compound C of (55)) were both supplied by Chromadex inc. 2952 S. Daimler St.

Santa Ana California. Protodioscin was also isolated from the above preparation of fenugreek as peak 2 conforming to published NMR spectra of protodioscin

Biological activity of Trigoneoside IVa, glycoside F, protodioscin and shatavarin IV

Cell-free assay

Heart lysate from BB rats were incubated in the presence, and absence of 20ng/ml of each compound. After 1h incubation at 37°C, the activity of core 2 GlcNAc-T was measured, and expressed as pmoles/h/mg protein. The results are the mean of 3-5 separate experiments. The results are shown in Figure 15a

Trigoneoside IVa, its 25(R) isomer glycoside F and shatavarin IV are highly active inhibitors of Core 2 GlcNAc-T in cell free assays, whilst protodioscin, in which the glucose at the 4 position is replaced by rhamnose, is not active.

Cell based assay

Human leukocytes (U937 cells) were exposed to 8 pg/ml human recombinant TNF-alpha in the presence and absence of 20 ng/ml of the test compound. After 24h incubation, the activity of core 2 GlcNAc-T was measured, and expressed as pmoles/h/mg protein. The results are shown in figure 15b.

Trigoneoside IVa, and glycoside F are highly active inhibitors of Core 2 GlcNAc-T in cell free assays, whilst protodioscin is not active.

The core 2 GlcNAc-T inhibitor trigoneoside IVa and diabetic retinopathy

It has been found that elevated glucose levels significantly increase the activity of core 2 GlcNAc-T in cultured bovine retinal vascular cells, namely capillary pericytes (BRP) and capillary endothelial cells (BREC) (Figure 13). Near confluent cultures were exposed to normal glucose (N, 5.8 mM) and high glucose (G, 15 mM) for 24 hours at 37°C. The cells were lysed and the activity of core GlcNAc-T measured in cell lysates.

It has further been demonstrated that fenugreek seed extract has the potential to reverse glucose-induced toxicity (Figure 14) in cultured bovine retinal capillary pericytes (BRP) and endothelial cells (BREC). Cells were exposed to normal (N, 5.8 mM) and high 15 glucose (G, 25 mM) in the presence (N-F, G-F) and absence (N, G) of the fenugreek seed extract. After 4 days incubation, the number of viable cells was

determined using a haemocytometer and trypan blue exclusion. It was found that fenugreek seed extract indeed reverses glucose-induced toxicity in cultured bovine retinal capillary pericytes and endothelial cells. However, it is not established yet whether fenugreek seed extract reverses glucose-induced toxicity by normalising the activity of core 2 GlcNAc-T.

This protection of retinal vascular cells fenugreek seed extract is significant, because damage to retinal vascular cells is a hallmark of early diabetic retinopathy. Diabetic retinopathy in humans is mainly a vascular disease, primarily affecting the capillaries. The first ultrastructural and microscopic changes reported are retinal capillary basement membrane thickening and pericyte degeneration, both of which compromise the integrity of the capillary wall. Pericyte degeneration leaves lightly stained compartments in the basement membrane sheath called pericyte "ghosts". Damage to both pericytes and endothelial cells leads to the formation of acellular capillaries.

Treatment

Medicaments comprising the compounds of the formula I described herein can be administered by oral or parenteral routes, including intravenous, intramuscular, intraperitoneal, subcutaneous, transdermal, airway (aerosol), rectal, vaginal and topical (including buccal and sublingual) administration. For oral administration, the compounds of the invention will generally be provided in the form of tablets or capsules, as a powder or granules, or as an aqueous solution or suspension.

Tablets for oral use may include the active ingredients mixed with pharmaceutically acceptable excipients such as inert diluents, disintegrating agents, binding agents, lubricating agents, sweetening agents, flavouring agents, colouring agents and preservatives. Suitable inert diluents include sodium and calcium carbonate, sodium and calcium phosphate, and lactose, while corn starch and alginic acid are suitable disintegrating agents. Binding agents may include starch and gelatine, while the lubricating agent, if present, may be magnesium stearate, stearic acid or talc. If desired, the tablets may be coated with a material, such as glyceryl mono stearate or glyceryl distearate, to delay absorption in the gastrointestinal tract. Capsules for oral use include hard gelatine capsules in which the active ingredient is mixed with a solid diluent, and soft gelatine capsules wherein the active ingredients is mixed with water or an oil such as peanut oil, liquid paraffin or olive oil

Formulations for rectal administration may be presented as a suppository with a suitable base comprising, for example, cocoa butter or a salicylate.

Formulations suitable for vaginal administration may be presented as pessaries, tampons, creams, gels, pastes, foams or spray formulations containing in addition to the active ingredient such carriers as are known in the art to be appropriate.

For intramuscular, intraperitoneal, subcutaneous and intravenous use, the compounds of the invention will generally be provided in sterile aqueous solutions or suspensions, buffered to an appropriate pH and isotonicity. Suitable aqueous vehicles include Ringer's solution and isotonic sodium chloride. Aqueous suspensions according to the invention may include suspending agents such as cellulose derivatives, sodium alginate, polyvinylpyrrolidone and gum tragacanth, and a wetting agent such as lecithin. Suitable preservatives for aqueous suspensions include ethyl and n-propyl p-hydroxybenzoate.

The fenugreek seed extracts and core 2 GlcNAc-T inhibitors of the present invention may also be presented as liposome formulations.

In general a suitable dose will be in the range of 0.01 to 10 mg per kilogram body weight of the recipient per day of the core 2 GlcNAc-T inhibitor, preferably in the range of 0.2 to 1.0 mg per kilogram body weight per day. The desired dose is preferably presented once daily, but may be dosed as two, three, four, five, six or more sub-doses administered at appropriate intervals throughout the day. These sub-doses may be administered in unit dosage forms, for example, containing 10 to 1500 mg, preferably 20 to 1000 mg, and most preferably 50 to 700 mg of active ingredient per unit dosage form.

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